Volatiles from Biofumigant Plants have a Direct Effect on Carpogenic Germination of Sclerotia and Mycelial Growth of *Sclerotinia sclerotiorum*

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Abstract (200 words)

Aims Sclerotia of *Sclerotinia sclerotiorum* survive in soil and germinate to produce apothecia which release airborne ascospores. Current control methods rely predominantly on the use of fungicides to kill ascospores. The aim of this research was to identify potential biofumigation treatments which suppress sclerotial germination, providing a potential alternative and long-term approach to disease management.

Methods Microcosm and *in vitro* experiments were conducted using dried and milled plant material from six different biofumigant crop plants to determine effects on carpogenic germination of sclerotia and mycelial growth of *S. sclerotiorum*.

Results All biofumigant plants significantly reduced germination of *S. sclerotiorum* sclerotia in the microcosm experiments, but were less effective against larger sclerotia. *In vitro* experiments showed a direct effect of biofumigant volatiles on both the mycelial growth of *S. sclerotiorum*, and carpogenic germination of sclerotia, where the most effective treatment was *B. juncea* 'Vittasso'.

Conclusions It was clear from this study that biofumigant crop plants have potential as part of an integrated disease management system for control of *S. sclerotiorum*. The microcosm experiments described here provide a straightforward and reliable screening method for evaluating different biofumigants for activity.

Keywords (4-6) *Sclerotinia sclerotiorum*; biofumigation; glucosinolate; isothiocyanate; *Brassica*

Introduction

Sclerotinia sclerotiorum (Lib.) de Bary is a plant pathogenic fungus with a world-wide distribution (Purdy 1979) and a large host range of over 400 plant species (Boland and Hall 1994). The pathogen affects many economically important crops including lettuce, oilseed rape, beans, peas, potatoes and carrots (Hegedus and Rimmer 2005). The long term survival structures produced by *S. sclerotiorum* are small black resting bodies called sclerotia (Willetts and Wong 1980) which when brought close to the soil surface germinate carpogenically to produce mushroom-like apothecia. These then release air-borne ascospores which infect plants, on which further sclerotia are eventually formed and returned to the soil (Bolton et al. 2006). An apothecium can produce up to sixteen hundred ascospores per hour, equivalent to 7.6×10^5 ascospores over a twenty day period (Clarkson et al. 2003). Under the correct conditions, ascospores germinate and use senescent tissues such as petals and leaves as a nutrient source to enable subsequent infection of the host plant (Bardin and Huang 2001).

The longevity of sclerotia is variable, and is influenced by many factors including the time and depth of burial (Duncan et al. 2006), and soil type (Merriman 1976). Under favourable conditions a large proportion of sclerotia can survive at least three years in the soil (Ćosić et al. 2012). The number of sclerotia produced by *S. sclerotiorum* on different plant tissues is also variable and is an important factor in determining the inoculum levels in soil following an infected crop. For instance, an infected cabbage head was found to produce 250 to 500 sclerotia, (Leiner and Winton 2006) while an infected carrot root produced up to 30 (Jensen et al. 2008).

In the absence of resistant crop cultivars, the main control method for Sclerotinia disease is the application of fungicides which kill ascospores before they infect plants. However, some of the effective active ingredients in fungicides currently used routinely against Sclerotinia disease such as boscalid, carbendazim, cyprodinil, fludioxonil (Matheron and Porchas 2008), azoxystrobin and difenoconazole are classed as medium to high risk for resistance (McQuilken 2011). In addition, there is a general desire to reduce such fungicide inputs, and hence approaches to reduce the viability of *S. sclerotiorum* sclerotia are required. Various non-organic soil amendments have been shown to inhibit sclerotial germination, such as potassium bicarbonate (Ordonez-Valencia et al. 2009) and calcium cyanamide (Perlka®) (Huang et al. 2006). Other control approaches aimed at reducing the number of sclerotia in soil include crop rotation, soil solarisation and application of the biological control agent *Coniothyrium minitans* (Jones et al. 2014; Mueller et al. 2002; Swaminathan et al. 1999).

The term ‘biofumigation’ describes the process of using specific *Brassica* crops which are macerated and incorporated into the soil to suppress pests and diseases (Kirkegaard et al. 1993). Many *Brassica* spp. produce significant levels of glucosinolates (GSL), which are sulphur-containing secondary plant

metabolites (Mithen 2001). These are held in plant cells separately from the enzyme myrosinase and in themselves are not fungitoxic (Manici et al. 1997). GSLs are classed as β-thioglucoside N-hydroxysulfates, with a side chain and a sulphur-linked β-d-glucopyranose moiety (Fahey et al. 2001) and are divided into aliphatic, aromatic and indole GSLs according to the type of side chain (Fenwick et al. 1983). GSL content can vary in the same crop grown in different years (Bangarwa et al. 2011) and between tissues within a single plant, such as between leaves and seeds (Gupta et al. 2012), or between leaves and flower buds. When a plant cell is ruptured as a result of pest or pathogen attack, or mechanical wounding, the GSLs and myrosinase come into contact and are hydrolysed in the presence of water to release various products, including isothiocyanates (ITCs) (Vig et al. 2009) which have a wide range of biocidal characteristics (Kurt et al. 2011) and are acutely toxic to several pathogenic fungi (Chew 1987). The definitive mode of action of ITCs inhibiting fungal growth and other microorganisms is uncertain, but some hypotheses include inactivation of intracellular enzymes by oxidative breakdown of sulphur bridges (Zsolnai 1966); inhibition of the coupling between the phosphorylation reactions and electron transport, therefore hindering the ATP synthesis (Kojima and Oawa 1971); inhibition of metabolic enzymes by the thiocyanate radical (Banks et al. 1986); inhibition of Tumour Necrosis Factor proteins, involved in extrinsic apoptosis, or induction of intrinsic apoptosis, depending on the type of ITC (Molina-Vargas 2013).

Due to the volatility of ITCs, biofumigant *Brassica* crops have been used to suppress a range of soilborne pests and pathogens (Kirkegaard et al. 1993) as well as some nematodes (Matthiessen and Kirkegaard 2006). Some of these natural ITCs have also been demonstrated to be more effective against some fungi than the synthetic ITC soil fumigant metam sodium (methyl isothiocyanate) (Sarwar et al. 1998). Few studies have investigated the use of biofumigants for control of Sclerotinia disease, and results have been inconsistent. Porter et al. (2002) reported that BQ Mulch (a blend of *B. napus* varieties) and *B. juncea* 'Fumus' significantly reduced the levels of disease on field grown lettuce crops caused by *S. minor*, whereas Bensen et al. (2009) observed significant short-term reductions in *S. minor* on lettuce when using *Sinapis alba* 'Ida Gold' and *B. juncea* 'ISCI 61' mustards. However, Geier (2009) found no significant effect of *B. juncea* 'Pacific Gold' on the carpogenic germination of *S. sclerotiorum* sclerotia in a polytunnel experiment.

Laboratory approaches have demonstrated the potential of biofumigant plants and ITCs to reduce mycelial growth of *S. sclerotiorum* or mycelial germination of sclerotia. For instance, Kurt et al. (2011) found synthetic (pure) ITCs significantly reduced mycelial growth of *S. sclerotiorum* and sclerotial viability *in vitro*, with six of the seven ITCs tested providing complete suppression of sclerotial germination. *B. juncea* was found to be the only cruciferous plant tested to affect sclerotial viability of *S. sclerotiorum* in another study, delaying myceliogenic germination by seven days, although after ten days approximately 80 % of the sclerotia germinated (Smolinska and Horbowicz

1999). However, whilst Rahimi et al. (2013) and Ojaghian et al. (2012) also found *B. juncea* varieties to be the most effective in inhibiting mycelial growth, other studies reported *B. oleracea* var. *caulorapa* and *B. nigra* to inhibit mycelial growth of *S. sclerotiorum* by 89.5 % (Fan et al. 2008) and 100 %, respectively (Rahmanpour et al. 2013).

None of the studies so far have directly assessed the effect of biofumigants or ITCs on carpogenic germination of *S. sclerotiorum* sclerotia, which is the principal mode of germination and inoculum production in the field. It is also clear that there is variation in the efficacy of different biofumigant plants inhibition of mycelial growth of *S. sclerotiorum*, which could in part be related to the variation in the level of GSLs and the potential for conversion to ITCs. However, few of studies quantify the GSL content of the *Brassica* species used, which limits the ability to make comparisons and also means that any observed effects cannot potentially be related to GSL levels, and could therefore be due to release of other compounds unrelated to GSL hydrolysis and ITC release. Moreover, incorporation of biofumigant crops may also have indirect effects through changes in populations of antagonistic organisms (Matthiessen and Kirkegaard 2006) so it is important to know if high GSL levels are present and ITC release is a possible mode of action. Reversed-phase high performance liquid chromatography (HPLC) provides an established method for analysis and quantification of GSLs as it can detect them in both intact and desulfated forms (Tsao et al. 2002) and hence can be used as an indirect measure of the biofumigation potential of plants (Wathelet et al. 2004).

The main aim of this study was to assess six biofumigant *Brassica* crop plant species for their ability to reduce the carpogenic germination of *S. sclerotiorum* sclerotia and hence production of apothecia. Experiments were also carried out to determine if this effect was due to the direct effect of volatiles released from the plant material and if there was a similar inhibitory effect on the mycelial growth of the pathogen. In addition, levels of GSLs were quantified in the plant material and minimum concentrations required to reduce mycelial growth by 50 % calculated for selected biofumigant plants.

Materials and Methods

Production of plant material

All biofumigant crop plants (*B. juncea, B. napus, Eruca sativa, Raphanus sativus* and *Sinapis alba*) were grown in 7.5 L pots, [five seeds per pot, Levington M2 compost (Scotts)] in a glasshouse with supplemental lighting and a venting temperature of 20 °C, or in a polytunnel, and whole plants harvested within two weeks of first flowering (approximately eight weeks after sowing). Harvested plants were immediately placed in an oven at 80 °C for 24 hours, then milled to a fine powder (Brook Compton Series 2000 mill, UK) and finally stored in sealed plastic bags at -20 °C. Repeat crop sowings were carried out in the glasshouse on 20/12/2011, 8/2/2012, 6/3/2012, 26/3/2012 and 12/4/2012, with harvesting on 16/2/2012, 4/4/2012, 1/5/2012, 29/5/2012 and 27/6/2012, respectively. Crops were sown in the polytunnel on 28/5/2012, 26/7/2012 and 20/8/2012, with harvesting on 25/7/2012, 12/9/2012 and 5/11/2012.

HPLC analysis of glucosinolates

Quantification of the main GSL in each biofumigant crop plant species was carried out using a simplified extraction and HPLC method adapted from Tsao et al. (2002). All extractions were carried out on a random bulked sample of the total dried plant material produced at each harvest date. To extract GSLs, reverse osmosis (RO) water (100 ml) was brought to boiling point on a heating mantle in a round-bottomed flask with some anti-bumping granules (VWR International Ltd, UK). Dried and milled plant material (1 g) was then added and the mixture kept at boiling point for 30 min with use of a reflux condenser. The mixture was allowed to cool before filtration through a 25 µm syringe filter. HPLC analysis was then undertaken using a HP Agilent 110 series system with a UV diode array detector. Separations were at approximately 24 °C on a reverse-phased Zorbax SB-Aq 4.6 x 250 mm 5 µm column (Agilent Technologies, USA), with a running pressure of approximately 43 bar. An eluent of 0.025 M $CH_3CO_2NH_4$ (ammonium acetate) in acetonitrile (pH 6.75) was used with a pump rate of 1 ml min^{-1} and an injection volume of 20 µl. The gradient was increased from 99 % ammonium acetate to 50 % at six min, and then back to 99 % after 21 min, for a total run time of 26 min. The retention times of the GSLs varied from 3 to 8 min, with detection at 228 nm. To identify any breakdown of GSLs to ITCs, detection was also run at 242 nm, as at this wavelength ITCs show as a larger peak than the same peak detected at 228 nm (Tsao et al. 2002). Standards of pure glucosinolates (sinigrin, sinalbin, glucoraphenin, glucobrassicanapin and glucoerucin at1000 ppm and 100 ppm; Phytolab GmbH & Co, Germany) were run in between every three samples, and a 1000

ppm standard of pure allyl ITC (Sigma Aldrich, UK) was run once to verify the peak size difference between wavelengths.

Culturing of *Sclerotinia sclerotiorum* and production of sclerotia

Culturing of *S. sclerotiorum* and production of sclerotia was carried out as described by Clarkson et al. (2003). Actively growing cultures of *S. sclerotiorum* isolates L6, L17 and L44 (all originally isolated from lettuce, Petworth, Sussex, in 2005; Clarkson et al. 2013) were produced from sterile stock sclerotia of each isolate (stored at 5 °C or at -20 °C in potato dextrose broth (PDB; Formedium, UK) amended with 10 % glycerol (Sigma-Aldrich Company Ltd, UK)). Before *S. sclerotiorum* sclerotia were used in experiments those between 2 and 5 mm were selected by sieving, and 'conditioned' by placing in mesh bags (100 per bag) buried in a pasteurised loam based compost (John Innes No 1, J. Arthur Bowers, UK; autoclaved at 110 °C for 30 min) for 6 weeks at 5 °C, which ensured rapid and reliable germination (Clarkson et al. 2007). *S. sclerotiorum* isolate L6 was selected as a standard isolate for use in all experiments as it was known to be highly pathogenic, quick growing and consistently able to rapidly produce apothecia.

Microcosm experiments: effect of biofumigant treatments on carpogenic germination of *Sclerotinia sclerotiorum* sclerotia

Microcosm experiments were set up to test the effect of dried plant material from six biofumigant crop plants and three other soil treatments (Table 1) on the carpogenic germination of *S. sclerotiorum* sclerotia (isolate L6) when incorporated into pasteurised compost contained in 600 ml clear plastic boxes (Malsar Kest Ltd, UK). The method was developed in previous work examining germination of *S. sclerotiorum* sclerotia and results in reliable production of apothecia (Clarkson et al. 2007). Soil treatments of two products previously reported to have activity against *S. sclerotiorum* sclerotia, the fertiliser Perlka® (calcium cyanamide) (Huang et al. 2006) and the biological control Contans WG (a formulation of the mycoparasite *Coniothyrium minitans*) (Jones et al. 2014), as well as the biofumigant product Biofence (mustard meal pellets) were also tested. *B. napus* 'Temple', a commercially grown winter oilseed rape cultivar, was used as a low GSL 'control' treatment to compare with the six biofumigant plants. An untreated control (compost only) was also included.

Dried plant material of each biofumigant (grown in polytunnel, harvested 25/7/2012 and 12/9/2012) or soil treatment was mixed with pasteurised John Innes No. 1 compost (low moisture, approx. 15 % w/w) at the appropriate rate (Table 1) and 350 g of the mixture placed into the clear plastic boxes. Pre-conditioned *S. sclerotiorum* sclerotia (isolate L6, 2-4 mm size) were then laid out in a grid pattern (6 x 5) in each box before adding another 50 g of the compost and treatment mixture to cover the

sclerotia to a depth of approximately 1 cm. An appropriate amount of water was then added to achieve 30 % (w/w) moisture content and initiate production of ITCs from the dried plant material. Lids were immediately placed onto the boxes which were weighed before being placed in a controlled environment room at 15°C with white fluorescent lighting (14 h day). Four replicate boxes were set up for each treatment, arranged in a randomised block design with four rows and 11 columns. To reduce any loss of volatiles from the biofumigant treatments, four replicate boxes for a single treatment were set up together in their entirety, before moving on to the next. To maintain constant moisture levels, water was added to the compost every two weeks to bring the boxes back to their original weight. Germination of *S. sclerotiorum* sclerotia was recorded twice a week as emergence of stipes or apothecia.

The quantity of dried plant material incorporated into the compost in the experiments was based on the average biomass produced in the field across the different biofumigant crop plants as supplied by the relevant seed companies (50-100 t $ha^{-1}$for an incorporation depth of 15 cm), and calculating equivalent quantities required for a 600 ml box with an incorporation depth of 6.5 cm (depth of compost used in each box). The calculated fresh weight of plants to be used in the boxes was then converted to a dry weight equivalent by weighing out three fresh samples of each plant, oven drying at 80 °C for 24 hours and then reweighing the samples. This resulted in 6 g dried plant material being used per box as an equivalent of full field rate. Rates of the other soil treatments used were based on manufacturer's field recommendations (Table 1). Overall, three experiments were carried out at a full rate of biofumigant (6 g dried plant material per box) and three at half rate (3 g dried plant material per box). In all experiments, the other soil treatments (Biofence, Perlka®, and Contans WG) were tested at the full field rate equivalents (Table 1).

Microcosm experiments: effect of size of *Sclerotinia sclerotiorum* sclerotia on the efficacy of biofumigant treatments

Experiments were set up to test the effect of the biofumigant *B. juncea* 'Caliente 99' (full field rate equivalent, 6 g per box, crop harvested 12/9/2012 and 5/11/2012) on germination of different sized sclerotia from three *S. sclerotiorum* isolates, L6, L17 and L44. The sclerotia were produced on wheat grain as described before, but passed through sieves to separate them into the following three different size grades: large, >5.6 mm; medium, 4-5.6 mm; small, 2-4 mm. Microcosms were set up as before with three replicates of each sclerotial size grade for each isolate and a total of three experiments were carried out. An untreated control (compost only) was also included.

In vitro experiments: effect of biofumigant plants on mycelial growth of *Sclerotinia sclerotiorum*

In vitro experiments were carried out to test the direct effect of volatiles (potentially ITCs) released from the dried material of the six biofumigant crop plants and oilseed rape (grown in polytunnel, harvested 25/7/2012) on mycelial growth of *S. sclerotiorum* using a method adapted from Sexton et al. (1999). A 5 mm agar plug of actively growing mycelium from *S. sclerotiorum* isolate L6 was placed in the centre of a PDA plate, the Petri dish inverted, and the dried plant material (1 or 2 g) placed in the lid. Water was then added at a rate of 10 ml per 1 g dried plant material and the Petri dish immediately closed and sealed with Parafilm® M (Bemis Co. Inc., U.S.A.). An untreated control was also set up consisting of water only in the lid. All Petri dishes were placed at 15 °C in the dark and mycelial growth of *S. sclerotiorum* was assessed twice a day for four days by the measuring colony diameter (mm) along two perpendicular axes through the midpoint. There were five replicate plates for each treatment arranged in a randomised block design and the experiment was repeated three times for each rate of plant material.

Experiments were also carried out to determine the dose response and the effective dose to provide 50 % inhibition (ED50) of *S. sclerotiorum* colony growth for two biofumigant plants, *B. juncea* 'Caliente 99' (crop harvested 12/9/2012) and *S. alba* 'Brisant' (crop harvested 25/7/2012). This was carried out as described above, using a range of dried plant material quantities (0.25, 0.5, 0.75, 1, 1.25, 1.5, 1.75 and 2 g) and water at the rate of 10 ml per 1 g dried plant material.

In vitro experiments: effect of biofumigants on carpogenic germination of *Sclerotinia sclerotiorum* sclerotia

In vitro experiments were carried out to test the direct effect of volatiles (potentially ITCs) released from the six biofumigant plants and oilseed rape (grown in glasshouse, harvested 27/6/2012; grown in polytunnel, harvested 25/7/2012 and 12/9/2012) on carpogenic germination of *S. sclerotiorum* sclerotia. Pasteurised John Innes No. 1 compost (50 g, 30 % w/w moisture content) was placed in a 9 cm Petri dish and preconditioned *S. sclerotiorum* sclerotia from isolate L6 (20) laid out in a grid pattern and pressed flat into the compost. Each Petri dish was placed into a 1200 ml clear plastic box (Malsar Kest Ltd, UK), together with a separate round plastic dish (6 cm diameter, 3 cm depth) containing either 1 g or 2 g of the dried biofumigant plant material. Water (10 ml per 1 g dried plant material) was added and the lids immediately placed on the plastic boxes. Boxes were weighed before being incubated in a controlled environment room at 15 °C in the dark and the emergence of stipes or apothecia recorded once a week for 80 days. The compost in the Petri dishes was watered to bring them back to their original weight every two weeks. Control treatments consisted of boxes set up with just water in the separate dish. Four replicates for each treatment were arranged in a randomised block

design and the experiment was repeated three times for each rate of plant material. For two of the three experiments (for both 1 g and 2 g plant material) the dish of biofumigant material was removed after 80 days and monitoring of germination continued once a week for an additional four weeks, to assess whether the sclerotia were killed by the biofumigant treatments, or whether germination was suppressed.

Statistical analyses

All statistical analyses were carried out using Genstat® (13th edition, VSN International Ltd). For the experiments testing effect of biofumigants on germination of *S. sclerotiorum* sclerotia, the number germinating in the microcosm and *in vitro* experiments after 150 and 80 days, respectively were analysed using a Generalised Linear Model (GLM) and logistic regression, with fitted terms of replicate and treatment and an estimated dispersion parameter. Interpretations from the GLM and logistic regression were made by comparing t probabilities calculated with reference to the untreated control for each set of experiments. The number of sclerotia germinating (after 150 days) in the microcosm experiments testing the effect of sclerotial size on biofumigant efficacy were analysed using Analysis of Variance (ANOVA) with a blocking structure of trial x replicates; interpretations were carried out by comparing ANOVA treatment means using the approximate least significant difference values (LSD) at the 5 % level.

For the experiments testing effect of biofumigants on *S. sclerotiorum* mycelial growth *in vitro*, colony measurements over time were fitted to logistic curves and the rate of growth calculated. The percentage growth inhibition was calculated at the time the untreated controls had grown to the edge of the Petri dish (after 66-73 hours), as I = ((C-T)/C) x 100, where I = % inhibition of mycelial growth, C = colony size of untreated control, T = colony size with biofumigant treatment. ED50 values for the dose response experiments were calculated through Probit analysis (Finney 1971), with a fitted term of log10 dose +0.01. The logistic curve parameters and the percentage of mycelial growth inhibition were analysed using ANOVA with a blocking structure of replicates, and interpretations were carried out by comparing ANOVA treatment means using the approximate least significant difference values (LSD) at the 5 % level.

Results

HPLC analysis of glucosinolates

The HPLC analysis of the biofumigant plant material clearly showed peaks on the chromatograms at retention times comparable with the appropriate GSL standards used. As expected, all *B. juncea S. alba, R. sativus*, and *E. sativa* samples contained sinigrin, sinalbin, glucoraphanin and glucoerucin, respectively. There were no unexpected peaks detected in any of the samples which would indicate the presence of a significant quantity of any other GSL.

The highest level of the GSL sinigrin was found in *B. juncea* 'Pacific Gold' harvested on 04/04/12 (33.29 µmol/g^{-1} dw, Table 2). Of the *B. juncea* varieties, the lowest levels of sinigrin at each harvest date were found in *B. juncea* 'Vittasso', ranging from 2.16 µmol/g^{-1} dw for the crop harvested 05/11/12 to 19.65 µmol/g^{-1} dw for the crop harvested 04/04/12. The highest level of sinalbin of 31.08 µmol/g^{-1} dw in *S. alba* 'Brisant' was found in the crop harvested 01/05/12, and the highest level of glucoerucin of 25.66 µmol/g^{-1} dw in *E. sativa* 'Nemat' was found in the crop harvested 25/07/12. The highest level of glucoraphenin in *R. sativus* 'Terranova' of 17.73 µmol/g^{-1} dw was found in the crop harvested 29/05/12. Very low levels of sinigrin were found in *B. napus* 'Temple' in plants from two of the eight harvest dates (27/06/12 and 05/11/12) and no other GSL was detected by the HPLC analysis. No conversion of GSLs to ITCs resulting from either the extraction method, or occurring from elution during the HPLC analysis, was observed for any of the samples using detection at 242 nm.

Microcosm experiments – effect of biofumigant treatments on carpogenic germination of *Sclerotinia sclerotiorum* sclerotia

GSL levels of the crops used in the microcosm experiments (harvested 25/7/2012 and 12/9/2012) are shown in Table 2. All the biofumigant plants and *B. napus* 'Temple' significantly reduced germination of *S. sclerotiorum* sclerotia in comparison with the untreated control after 150 days at both half and full rates (Fig. 1). Of the biofumigant plants, *R. sativus* 'Terranova' resulted in the greatest reduction in germination with a mean total germination of 6.8 sclerotia compared to 22.1 in the untreated control (full rate). The low glucosinolate *B. napus* 'Temple' reduced mean germination to 9.3 sclerotia (full rate). Of the positive controls (used at full field rates in all experiments) the greatest reduction in germination was observed with Perlka® where a mean total germination of 1.6 sclerotia was recorded. There were generally only small differences between the germination observed in the half rate experiments and the full rate experiments for the biofumigant treatments.

Microcosm experiments – effect of size of *Sclerotinia sclerotiorum* sclerotia on the efficacy of biofumigant treatments

In experiments testing the effect of the biofumigant *B. juncea* 'Caliente 99' on germination of different sized *S. sclerotiorum* sclerotia (isolates L6, L17, L44), the mean number of apothecia produced per sclerotium by the different sizes of sclerotia ranged from 0.9 (L17 small sclerotia) to 3.1 (L6 large sclerotia) with the large sclerotia producing the most apothecia overall across all isolates (Table 3). GSL levels of the crops used (harvested 12/9/2012 and 5/11/2012) are shown in Table 2. For *S. sclerotiorum* isolate L6 there was a significant reduction in germination for sclerotia treated with *B. juncea* 'Caliente 99' and the untreated sclerotia for all three sizes ($P<0.05$; Fig. 2). The efficacy of biofumigation was greatest for the medium sized sclerotia, reducing germination by 60.8 %, and lowest in the large sclerotia where germination was reduced by 37.1 % (Table 3). For *S. sclerotiorum* isolate L17, there were again significant differences between the treated and untreated sclerotia for all three sizes ($P<0.05$; Fig. 2) and the efficacy of biofumigation was greatest for the small sclerotia (92.4 % reduction in germination) and lowest for the large sclerotia (75.3 % reduction in germination, Table 3). For isolate L44 there was no significant difference between the treated and untreated sclerotia for all three sizes, but there was very low germination in the untreated controls (Fig. 2). Across all *S. sclerotiorum* isolates the efficacy of biofumigation was 72-75 % for small and medium sclerotia, compared to 57.5 % for the large sclerotia (Table 3).

In vitro* experiments: effect of biofumigant plants on mycelial growth of *Sclerotinia sclerotiorum

For the *in vitro* experiments testing the direct effect of volatiles released from plant material (GSL levels of the crops used harvested on 25/7/2012 are shown in Table 2) on mycelial growth of *S. sclerotiorum* L6, rate of growth was significantly ($P<0.05$) reduced for *B. juncea* 'Pacific Gold', 'Caliente 99' and 'Vittasso', *S. alba* 'Brisant' and *B. napus* 'Temple' using 1g plant material, with with rates ranging from 0.9 to 1.5 mm h^{-1} compared to 1.6 mm h^{-1} for the untreated control (Table 4).. In experiments using 2 g plant material, the rate of mycelial growth was only significantly reduced for *B. juncea* 'Caliente 99', 'Vittasso' and 'Pacific Gold' ($P<0.05$), with growth rates ranging from 0.5 to 1.3 mm h^{-1} compared to 1.5 mm h^{-1} for the untreated control (Table 4).

After 73 hours (the time at which the mycelial growth of the untreated *S. sclerotiorum* control reached the edge of the Petri dish) there were significant differences in the percentage reduction of mycelial growth compared to the untreated control in experiments using 1 g and 2 g plant material (Fig. 3). The greatest inhibition was for *B. juncea* 'Caliente 99', (72.8 % for 1 g plant material and 85.3 % for 2 g plant material) and the *B. juncea* varieties inhibited mycelial growth more than any of the other biofumigant crop plants. The lowest growth inhibition (14.6 %) was observed for *R. sativus*

'Terranova' at a rate of 2 g. *B. juncea* 'Pacific Gold' and 'Caliente 99' were the only treatments which resulted in a greater inhibition of mycelial growth at the higher rate of 2 g compared to 1 g plant material.

In the experiments carried out to determine the dose response and ED50 for *B. juncea* 'Caliente 99' and *S. alba* 'Brisant', there were clear differences in the inhibition of mycelial growth of *S. sclerotiorum* isolate L6 after 72 hours when treated with the different quantities of these biofumigant crop plants. When the data was subjected to Probit analysis, the ED50 for *B. juncea* 'Caliente 99' was calculated as 1.86 g (equivalent to 33.44 µmol sinigrin), whereas for *S. alba* 'Brisant' the ED50 was 6.31 g (equivalent to 96.54 µmol sinalbin (Fig. 4)).

In vitro Experiments: Effect of Biofumigants on Carpogenic Germination of *Sclerotinia sclerotiorum* Sclerotia

In the experiments carried out to test the direct effect of volatiles released from biofumigant plants (GSL levels of the crops used harvested on 27/6/2012, 25/7/2012 and 12/9/2012 are shown in Table 2) on carpogenic germination of *S. sclerotiorum* sclerotia, there was limited or no reduction in the germination of *S. sclerotiorum* sclerotia using only 1 g plant material. However, all treatments significantly reduced germination using 2 g plant material (Fig. 5), the most effective being *B. juncea* 'Vittasso' with a mean germination of 3.8 sclerotia after 80 days compared to 19.8 sclerotia for the untreated control. *B. juncea* 'Pacific Gold' was the least effective treatment in the 2 g experiments with a mean germination of 14.2 sclerotia while *B. napus* 'Temple' reduced germination to a mean of 7.3 sclerotia. The latter was also the most effective treatment in the experiments with 1 g plant material with a mean of 12.3 sclerotia germinating (Fig. 5).

For the four experiments where the biofumigant treatment was removed and germination assessed for the following four weeks, there was further germination in all treatments except for *R. sativus* 'Terranova' at the 2 g rate (Fig. 6). The greatest additional germination was observed for *B. napus* 'Temple' at the 1 g rate, with a mean of 4.1 sclerotia germinating after removal of the treatment. The least additional germination was observed for *B. juncea* 'Vittasso' at the 2 g rate, with a mean of 0.1 sclerotia. For *B. juncea* 'Vittasso', *R. sativus* 'Terranova', *E. sativa* 'Nemat' and *B. napus* 'Temple' there was more germination after removal of the 1 g treatments than the 2 g treatments (Fig. 6).

Discussion

There have been very few studies that have examined the effects of biofumigant plants on the carpogenic germination of sclerotia of *S. sclerotiorum*. The results presented here clearly demonstrated that biofumigant treatments based on dried plant material from *B. juncea* 'Caliente 99', *B. juncea* 'Pacific Gold', *B. juncea* 'Vittasso', *S. alba* 'Brisant', *R. sativus* 'Terranova', and *Eruca sativa* 'Nemat' all reduced carpogenic germination of *S. sclerotiorum* sclerotia. *In vitro* experiments also showed that, for *B. juncea* cultivars, this is likely to have been caused directly by volatiles, most likely ITCs, released from the plant material. This is the first time that volatiles derived from crops bred specifically for biofumigation have been shown to have a direct effect on the carpogenic germination of *S. sclerotiorum* sclerotia.

Dried milled plant biofumigant material was used throughout this study because, for the microcosm experiments, initial tests with fresh material had resulted in waterlogging of the compost as the material broke down which resulted in rotting of the sclerotia. This approach is likely to maximise the release of ITCs by ensuring complete cell disruption, preserving the GSLs (as evidenced by the HPLC analysis) and promoting distribution of myrosinase. The dried material is also easily stored, and can be used for multiple experiments, hence reducing variation in bioassay results. GSL content following the drying and milling process (2.2 - 33.3 µmol/g^{-1} dw for sinigrin and 5.6 - 31.0 µmol/g^{-1} dw for sinalbin depending on variety and time of harvest), were within the range of 0.2 - 44.9 µmol/g^{-1} dw reported by Kirkegaard and Sarwar (1998) across 80 *Brassica* spp. and comparable with the ranges of 27 – 66 µmol/g^{-1} reported by Antonious et al. (2009) for 10 *Brassica* spp. and one *E. sativa*. The activity of the dried biofumigant plant material observed in this study across multiple assays with *S. sclerotiorum*, strongly suggests that both GSLs and myrosinase were sufficiently preserved during the processing of the plant material to enable ITC production, although further chemical analysis would be needed to unequivocally confirm this.

HPLC analysis showed a wide variation in GSL content in the plants used in the experiments, both between varieties of the same species, and within varieties harvested at different times of year. For example, differences in GSL content of plant material used in the microcosm experiments harvested on 25/07/12 and 12/09/12 were in the range 0.6 to 5.1 µmol/g dw. This highlights the need to measure GSLs in order to potentially explain variation in levels of activity as suggested by Matthiessen and Kirkegaard (2006), although statistical analysis of the results for each assay here showed no significant differences in efficacy of treatments between replicated experiments. This suggests that it is the type of GSL, and therefore the resulting ITC, which is more important than the quantity of GSL when screening potential biofumigant plants for efficacy against specific pathogens.

The microcosm experiments reported here represent a more robust, reproducible and realistic way of screening different biofumigants for their ability to suppress the carpogenic germination of *S. sclerotiorum* sclerotia than other methods reported previously. Most other studies, e.g. Smolinska and Horbowicz (1999), have determined the viability of *S. sclerotiorum* sclerotia by assessing myceliogenic germination on agar rather than carpogenic germination in compost or soil, despite the latter being the predominant type of germination in the field. However, the method using dried milled plant material here to conserve GSLs and optimise ITC production was aimed at identifying biofumigant plants with this direct mode of action rather than through the stimulation of beneficial microorganisms which has been suggested as a secondary mechanism of activity (Matthiessen and Kirkegaard, 2012). Whether the approach is a good indicator of the performance of biofumigants against *S. sclerotiorum* in the field, where GSL levels and ITC production are likely to be much lower, still requires evaluating. Nevertheless, the microcosm experiments clearly demonstrated the potential biofumigation effect of different plants, all of which reduced carpogenic germination of *S. sclerotiorum* sclerotia to some degree, with *R. sativus* 'Terranova' showing the greatest effect. There are no directly comparable studies but in a study by Bomford (2009), fresh plant material of *B. juncea* 'Pacific Gold' incorporated into soil failed to reduce carpogenic germination of *S. sclerotiorum* sclerotia, although they were only exposed to the biofumigant for three days. In contrast, Ojaghian et al. (2012) found that growing and incorporating *B. juncea* into a field as a green manure significantly reduced disease incidence of *S. sclerotiorum* on potatoes.

With the exception of Contans WG (*Coniothyrium minitans*) in the full rate set of experiments, all the positive control treatments used in the microcosm experiments reduced carpogenic germination of *S. sclerotiorum* sclerotia significantly, with Perlka® providing the greatest reduction of all the treatments. Similarly, Huang et al. (2006) found a reduction of between 65 and 87 % in production of apothecia by *S. sclerotiorum* sclerotia in bean fields when treated with Perlka® at a rate of 60 g/m^2. The reduced effectiveness of Contans WG is most likely due to the experimental methods, which meant there was not enough time for the inoculum concentration of *Coniothyrium minitans* to build up in the compost (Jones et al. 2003).

Different isolates of *S. sclerotiorum* have been shown to consistently produce different sizes of sclerotia (Akram et al. 2008; Li et al. 2008) and this has also been observed in *S. trifoliorum* (Vleugels et al. 2013). Hence in this study experiments were set up to assess the effect of sclerotial size on the efficacy of biofumigation. These showed that larger *S. sclerotiorum* sclerotia were less affected by *B. juncea* 'Caliente 99', with germination reduced by only 57 %, compared to the small and medium sclerotia where germination was reduced by over 70 %. Additionally, the larger *S. sclerotiorum* sclerotia germinated more consistently, which has also been reported by Dillard et al. (1995), producing twice the mean number of apothecia per sclerotia than the medium sclerotia, and three

times as many as the small sclerotia. These results suggest that biofumigation approaches may be less effective against larger sclerotia of *S. sclerotiorum*, which also have the greater inoculum potential. Similarly, Smolinska and Horbowicz (1999) reported that volatiles from *B. juncea* plant tissue caused a greater reduction in mycelial germination of small sclerotia produced by *Sclerotium cepivorum* than the large sclerotia produced by *S. sclerotiorum*, but this may also be due to different sensitivities of the species investigated. It is possible therefore that biofumigation against *Sclerotinia* spp. generally may be more effective against the smaller sclerotia produced by *S. trifoliorum* and *S. minor*, and less effective against the larger sclerotia produced by *S. subarctica* (Clarkson et al. 2010). Further work with a wider range of *S. sclerotiorum* isolates and with different species is needed to confirm this.

The *in vitro* experiments clearly demonstrated a direct effect of volatiles released from biofumigant plant material on carpogenic germination of *S. sclerotiorum* sclerotia, with all the biofumigant plants tested significantly reducing germination with 2 g material, including the low glucosinolate *B. napus* 'Temple'. Again, although there are no directly comparable studies, Bomford (2009) treated *S. sclerotiorum* sclerotia for 24 hours with extracted GSLs from 11 different *B. juncea* varieties. Carpogenic germination was assessed over six weeks after the sclerotia were removed from the treatments. All biofumigant plant varieties tested reduced germination, with *B. juncea* 'Pacific Gold' being the most effective. However, it was assumed that sclerotia were dead if they had not germinated after six weeks. It was observed in the present study that for some biofumigants up to four *S. sclerotiorum* sclerotia (of 20) germinated after treatments were removed, indicating some of the sclerotia were still viable and carpogenic germination was only suppressed. However, suppression may be a valuable mode of action in the field, and in order to assess viability the sclerotia would need to have been retrieved, bisected and plated onto PDA or water agar to see if they would germinate myceliogenically (Hao et al. 2003).

Another relevant study examined the volatile effects of a range of pure ITCs on carpogenic germination of *S. sclerotiorum* sclerotia *in vitro* and showed that all of them with the exception of phenyl and 2- phenylethyl ITC at their lowest concentrations (42 and 34 µmol L^{-1}, respectively) significantly inhibited the production of apothecia over a period of 10 to 14 weeks (Kurt et al. 2011). Butyl and benzyl ITC gave the greatest reduction in germination of 92.5 % at their highest concentrations (840 and 751 µmol L^{-1} correspondingly); these are aliphatic and aromatic ITCs, respectively. In the same study, allyl ITC reduced *S. sclerotiorum* germination by between 60 % and 75 %. By comparison, in this study the most effective treatment for reducing carpogenic germination *in vitro* (by 81 %) was *B. juncea* 'Vittasso', which would produce allyl ITC from the main GSL sinigrin. However, based on the HPLC analysis this variety had the lowest GSL content for each harvest date out of all the *B. juncea* varieties used in the experiments. This discrepancy is unlikely to be due differences in the efficacy of the conversion of GSLs to ITCs which has been shown to vary

between *Brassica* spp. when fresh material is incorporated into the soil, as no studies have compared conversion efficiences using dried milled plant material. For instance, using fresh plant material Morra and Kirkegaard (2002) found a lower conversion efficiency for a high GSL *B. juncea* variety (0.6 %) than for a low GSL variety (1.6 %). They also reported that freezing and thawing plant tissue increased ITC release efficiencies to 26 %

The most effective biofumigant plant for suppressing mycelial growth of *S. sclerotiorum* in this study was *B. juncea* 'Caliente 99'. Similarly, Ojaghian et al. (2012) reported that fresh macerated tissues of *B. juncea* 'Bresska' were the most effective in reducing mycelial growth of *S. sclerotiorum*, when compared to *B. napus* and *B. campestris*. Also using fresh macerated plant material, Larkin and Griffin (2007) reported that a *B. juncea* variety inhibited mycelial growth of *S. sclerotiorum* by 90.2 %, slightly higher than the 85.3 % found in the present study using a rate of 2 g dried plant material. Additionally, they found that *B. napus* 'Hyola 401' (low glucosinolate content) and *S. alba* 'Ida Gold' resulted in mycelial growth reductions of 20.4 % and 23.7 %, respectively, both lower than the reductions found in the present study of 28.6 % for *B. napus* 'Temple' and 33 % for *S. alba* 'Brisant'. However comparisons between the fresh plant material used by Larkin and Griffin (2007) and the dried plant material used in this study are difficult as they did not quantify GSL content.

In this study, the most effective biofumigant plant for inhibition of carpogenic germination of *S. sclerotiorum* sclerotia was *R. sativus* 'Terranova' for the microcosm experiments and *B. juncea* 'Vittasso' for the *in vitro* experiments. However, in the mycelial growth experiments, *B. juncea* 'Caliente 99' was the most effective. These differences are likely to be due to a combination of differences between contact phase and vapour phase effects, the different quantities of plant material used, i.e. 6 g in a full field rate microcosm experiment and a maximum of 2 g in the *in vitro* experiments, and a variation in susceptibility of the different tissues, i.e. sclerotia vs. mycelium. This variation in the ability of particular biofumigants to inhibit mycelial growth or carpogenic germination of *S. sclerotiorum* has also been observed by Kurt et al. (2011) who found that different pure aliphatic and aromatic ITCs were effective against mycelial growth or carpogenic germination, but that the efficacy depended on whether they were used as vapour or contact phases. Aliphatic ITCs (derived from GSLs such as sinigrin, glucoerucin and glucoraphenin) were more effective than aromatic ITCs (derived from GSLs such as sinalbin) in the vapour phase, with the opposite being true when the ITCs were incorporated into PDA in contact phase. This is in agreement with this study where the only biofumigant with a main GSL which would hydrolyse to produce an aromatic ITC was *S. alba* 'Brisant', which was more effective at inhibiting carpogenic germination in the microcosm experiments (contact phase) than in the *in vitro* carpogenic experiments (vapour phase). However, in the mycelial growth experiments it resulted in greater inhibition than some of the aliphatic GSL containing plants.

The HPLC analysis combined with the results from experiments suggests that non-glucosinolate derived volatiles were released from dried plant material from *B. napus* 'Temple' which reduced germination of *S. sclerotiorum* sclerotia. Previously it has been reported that synthetic pure butyl ITC, which is derived from the parent GSL gluconapin commonly found in some *B. napus* varieties and *B. juncea*, was the most effective ITC in inhibiting carpogenic germination of *S. sclerotiorum* sclerotia (Kurt et al. 2011). However, the HPLC analysis conducted here did not detect any GSLs in *B. napus* 'Temple', except for very small quantities of sinigrin. The finding of sinigrin was unexpected but low levels have been found before in *B. napus* leaves (Velasco et al. 2008). Similarly, Smolinska and Horbowicz (1999) detected no measurable amount of ITCs in two of the four *B. napus* varieties in their experiments. Another study found that a low glucosinolate *B. napus* variety reduced carpogenic germination of *S. sclerotiorum* sclerotia by 44 %, but did not affect myceliogenic germination (Dandurand et al. 2000). This is similar to the results from the present study, where *B. napus* 'Temple' was very effective in reducing carpogenic germination, but amongst the least effective in inhibiting mycelial growth. It was suggested by Bending and Lincoln (1999) that the biofumigant properties of *B. juncea* were due to the combined effect of small quantities of ITCs, and large quantities of less toxic non-glucosinolate derived volatile S-containing compounds, such as carbon-disulphide, dimethyl-disulphide, dimethyl-sulphide and methanethiol. These compounds, amongst others such as fatty-acid derivatives, were found in *B. napus* by Tollsten and Bergström (1988) so may be responsible for the observed inhibition of *S. sclerotiorum* here.

The results from the microcosm and *in vitro* experiments showed that volatiles released from biofumigation crops have a direct inhibitory effect on the mycelial growth and carpogenic germination of *S. sclerotiorum* sclerotia. The most effective biofumigation crop for inhibiting carpogenic germination varied depending on whether the volatiles released from the biofumigant crops were in direct contact with the sclerotia (as in the microcosm experiments) when the most effective crop was *Raphanus sativus* 'Terranova', or in the vapour phase (as in the *in vitro* experiments) when the most effective crop was *B. juncea* 'Vittasso'. Additionally, a different *B. juncea* cultivar ('Caliente 99') was most the effective in inhibiting mycelial growth, indicating differences in the susceptibility of the different fungal structures, i.e. mycelium vs. sclerotia. Therefore, using microcosm experiments is a more suitable method for establishing the most effective biofumigant crops against resting propagules of soil borne fungal pathogens, as it tests the biofumigants in an assay which most closely simulates how they would be applied in a field situation, against sclerotia rather than mycelium. The *in vitro* assays were, however, invaluable for establishing the direct effect of volatiles released from the biofumigant crops.

The reduction in the efficacy of biofumigation for larger *S. sclerotiorum* sclerotia highlights how this control method must be viewed as part of an integrated disease management system, and not a stand-alone treatment. The potential level of control achieved by biofumigation alone means it cannot be brought in as a direct replacement for chemical soil fumigants or foliar fungicide sprays, and should instead be used in combination with these and other approaches such as crop rotation, biological control and disease forecasting. Further research could investigate combining biofumigation with Contans WG to establish how they might work together.

The unexplained efficacy of the low GSL *B. napus* 'Temple' needs to be further investigated to understand which compounds are responsible for this cultivar inhibiting germination of *S. sclerotiorum* sclerotia. Once the mechanism behind this inhibition is understood, future research could look beyond only using high GSL *Brassica* spp. for biofumigation, and instead identify a range of crops with different inhibitory compounds. This would not only widen this area of research considerably, but also help to prevent pathogens such as *S. sclerotiorum* potentially becoming resistant to the volatiles released following the hydrolysis of GSLs.

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Table 1 Summary of biofumigant crop plant and other treatments used in microcosm experiments

Treatments	Main Glucosinolate	Full Field Rate [a] (g per box)	Half Field Rate [b] (g per box)
Brassica juncea 'Vittasso'	Sinigrin	6	3
Brassica juncea 'Pacific Gold'	Sinigrin	6	3
Sinapis alba 'Brisant'	Sinalbin	6	3
Brassica juncea 'Caliente 99'	Sinigrin	6	3
Raphanus sativus 'Terranova'	Glucoraphenin	6	3
Eruca sativa 'Nemat'	Glucoerucin	6	3
Brassica napus 'Temple'	Glucobrassicanapin / Sinigrin	6	3
Perlka® (calcium cyanamide)	n/a	0.43 [c]	n/a
Biofence (mustard meal pellets)	n/a	1.4 [c]	n/a
Contans WG (*Coniothyrium minitans*)	n/a	0.4 [c]	n/a
Untreated	n/a	0	0

[a] Based on achieving 100 tonnes per ha of biomass
[b] Based on achieving 50 tonnes per ha of biomass
[c] Based on field application rates of Perlka® (400 kg/ha), Biofence (3000 kg/ha) and Contans WG (8 kg/ha)

Table 2 Single glucosinolate levels (sinigrin, sinalbin, glucoraphenin and glucoerucin in µmol/g^{-1} dw) as determined by HPLC analysis for six different biofumigant plants and *Brassica napus* 'Temple' grown in a glasshouse (harvest dates 16/2/12, 4/4/12, 1/5/12, 29/5/12 and 27/6/12) and in a polytunnel (harvest dates 25/7/12, 12/9/12 and 5/11/12)

	Biofumigant Crop	***Brassica juncea* 'Vittasso'**	***Brassica juncea* 'Pacific Gold'**	***Brassica juncea* 'Caliente 99'**	***Brassica napus* 'Temple'**	***Sinapis alba* 'Brisant'**	***Raphanus sativus* 'Terranova'**	***Eruca sativa* 'Nemat'**
	Glucosinolate	**Sinigrin**	**Sinigrin**	**Sinigrin**	**Sinigrin**	**Sinalbin**	**Glucoraphenin**	**Glucoerucin**
Crop harvest date (glasshouse)	**16/02/2012**	7.65	16.64	12.04	0.00	12.56	7.13	15.34
	04/04/2012	19.65	33.29	32.64	0.00	16.54	15.45	16.46
	01/05/2012	17.60	28.07	24.00	0.00	31.08	15.77	16.26
	29/05/2012	8.74	23.97	20.93	0.00	23.26	17.73	14.23
	27/06/2012	6.33	16.21	26.52	0.35	14.46	12.74	25.19
Crop harvest date (polytunnel)	**25/07/2012**	5.64	12.52	18.55	0.00	15.30	9.28	25.66
	12/09/2012	3.81	11.65	17.98	0.00	10.17	13.02	23.85
	05/11/2012	2.16	7.12	5.82	1.32	5.57	7.36	16.33

Table 3 Percentage reduction in germination and mean number of apothecia per sclerotium after 150 days for three different sizes of *Sclerotinia sclerotiorum* sclerotia from isolates L6, L44 and L17 when treated with dried plant material of *Brassica juncea* 'Caliente 99' in microcosm experiments

***Sclerotinia sclerotiorum* Isolate**	**Size of Sclerotia[1]**	**% Reduction in Germination for Biofumigation Treatment**	**Mean Number of Apothecia per Sclerotium (for all treatments)**
L6	Small	54.6	1.1
L6	Medium	60.8	1.7
L6	Large	37.1	3.1
L17	Small	92.4	1.0
L17	Medium	91.3	1.3
L17	Large	75.3	2.9
L44	Small	100	0.9
L44	Medium	100	1.4
L44	Large	100	2.6
All isolates	Small	72.3	1.0
All isolates	Medium	74.9	1.5
All isolates	Large	57.5	2.9

[1]Small, 2-4 mm; medium, 4-5.6 mm; large, >5.6 mm.

Table 4 The effect of dried biofumigant plants and low glucosinolate *Brassica napus* 'Temple' on mycelial growth rate (based on fitted logistic curves) of *Sclerotinia sclerotiorum* isolate L6 at 15 °C in the dark over four days. * indicates significant difference to the untreated control at P<0.05

	Growth rate (mm h^{-1})			
Treatments	**1g**		**2g**	
Brassica juncea 'Pacific Gold'	0.92	*	1.28	*
Brassica juncea 'Vittasso'	1.36	*	1.20	*
Brassica juncea 'Caliente 99'	1.01	*	0.54	*
Raphanus sativus 'Terranova'	1.59		1.54	
S*inapis alba* 'Brisant'	1.44	*	1.59	
Eruca sativa 'Nemat'	1.51		1.47	
Brassica napus 'Temple'	1.46	*	1.52	
Untreated	1.63		1.54	
Significance at P<0.05	0.16		0.20	
d.f.	95		91	

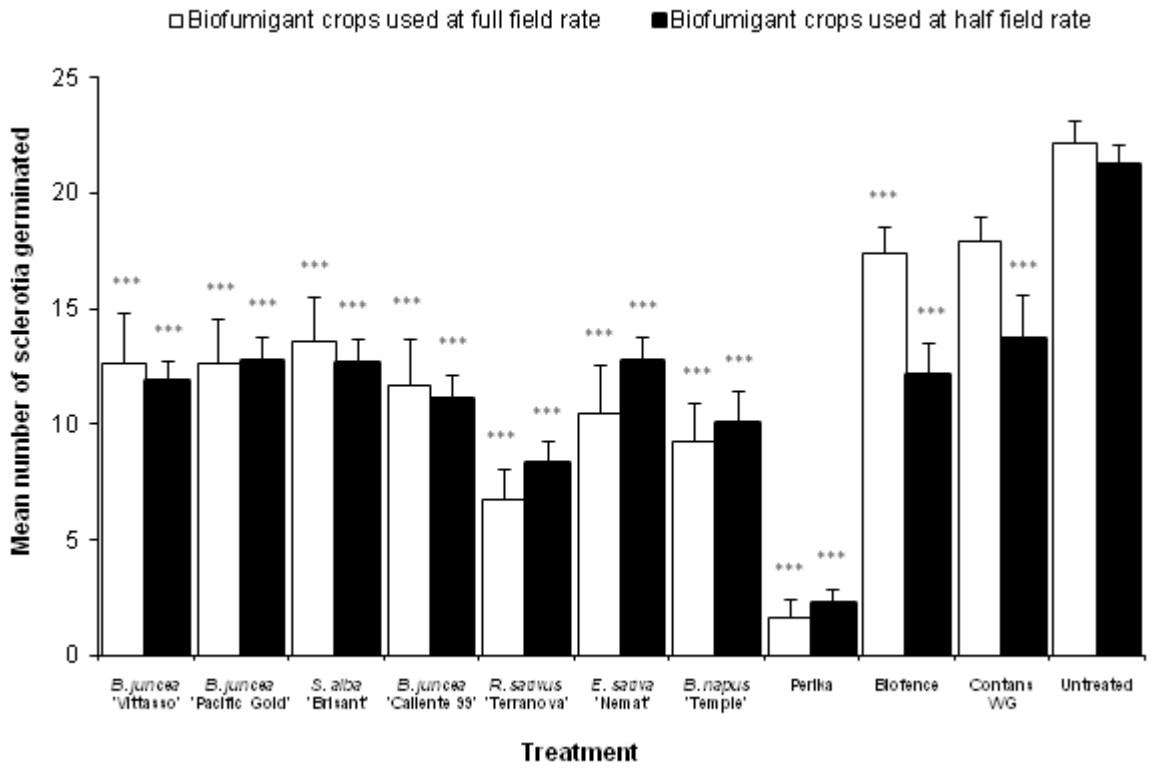

Figure 1. The effect of six biofumigant crop plants, the low GSL *Brassica napus* 'Temple', and the positive control treatments Perlka®, Biofence and Contans WG on the mean number of *Sclerotinia sclerotiorum* sclerotia germinated (out of 30) after 150 days at 15°C in microcosm experiments. Positive control treatments were used at full field rate in all experiments. Error bars indicate SEM. Significance of treatments compared to untreated control; ***, P< 0.001; **, P< 0.01; * P< 0.05

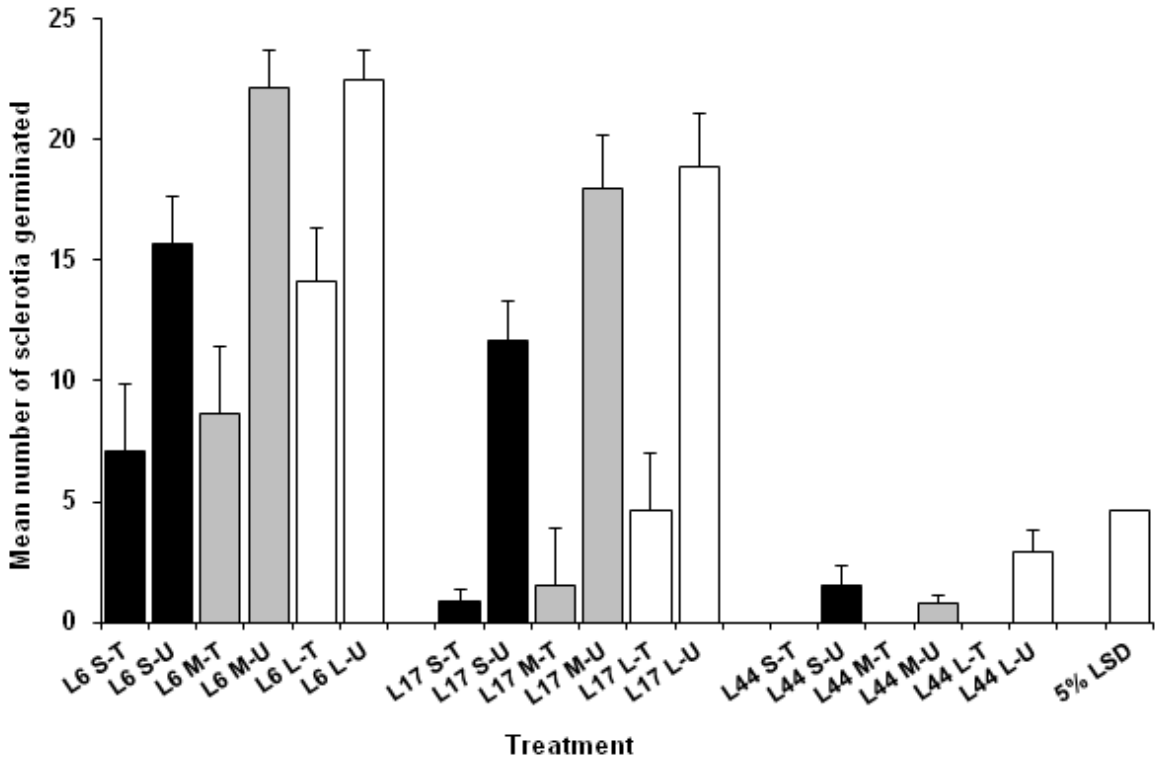

Figure 2. The effect of the biofumigant crop plant *Brassica juncea* 'Caliente 99'on the mean number of *Sclerotinia sclerotiorum* sclerotia germinated (out of 30) for three different sizes of sclerotia for isolates L6, L17 and L44 after 150 days at 15°C in microcosm experiments. S-T = small sclerotia treated, S-U = small sclerotia untreated, M-T = medium sclerotia treated, M-U = medium sclerotia untreated, L-T = large sclerotia treated, L-U = large sclerotia untreated. Error bars indicate SEM

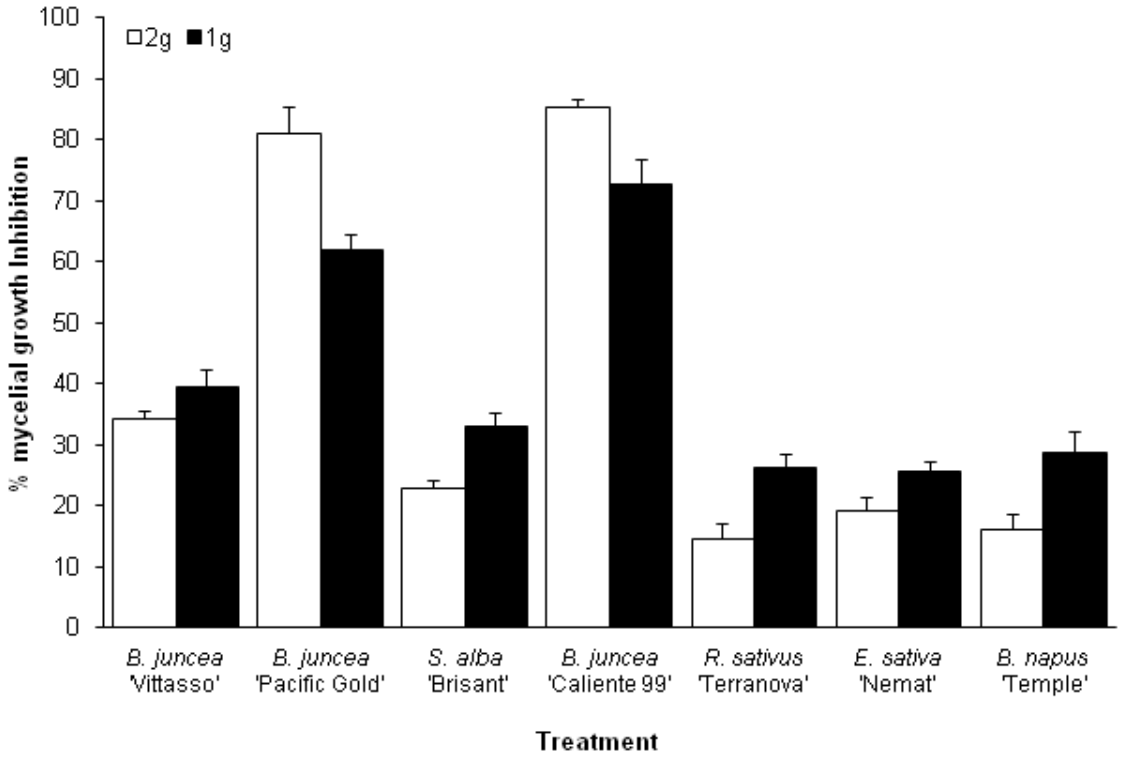

Figure 3. Effect of six biofumigant crop plants and *Brassica napus* 'Temple' on percentage mycelial growth inhibition of *Sclerotinia sclerotiorum* isolate L6 when compared to the untreated control after 73 hours at 20°C for 1 g and 2 g plant material. Error bars indicate SEM. LSD for 1 g = 7.21, LSD for 2 g = 6.32

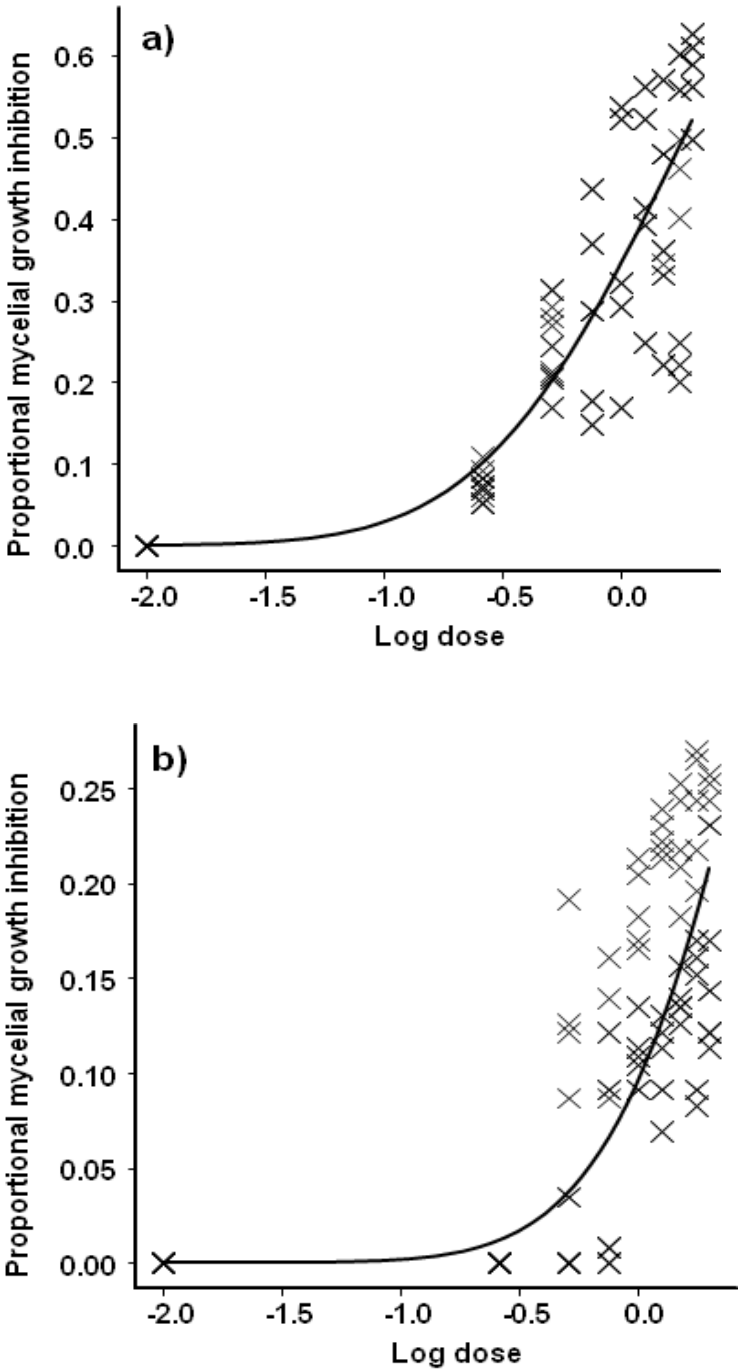

Figure 4. Fitted (line) and observed values (means across all replicates; crosses) of the proportional mycelial growth inhibition of *Sclerotinia sclerotiorum* isolate L6 to log dose of *Brassica juncea* 'Caliente 99' (A) and *Sinapis alba* 'Brisant' (B) dried and milled plant material after 72 hours

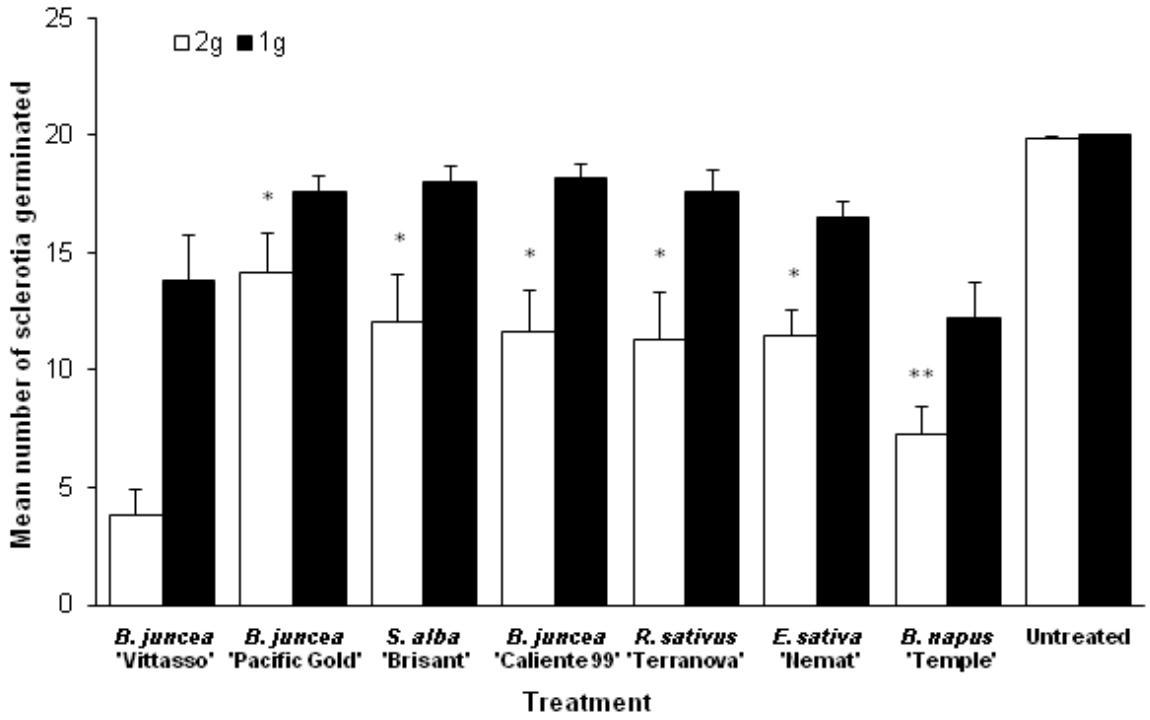

Figure 5. The effect of six biofumigant crop plants and *Brassica napus* 'Temple' on .the mean number of *Sclerotinia sclerotiorum* isolate L6 sclerotia germinated (out of 30) after 80 days at 15°C in the *in vitro* experiments for 1 g and 2 g plant material.. Error bars indicate SEM. Significance of treatments compared to untreated control; ***, P< 0.001; **, P< 0.01; * P< 0.05

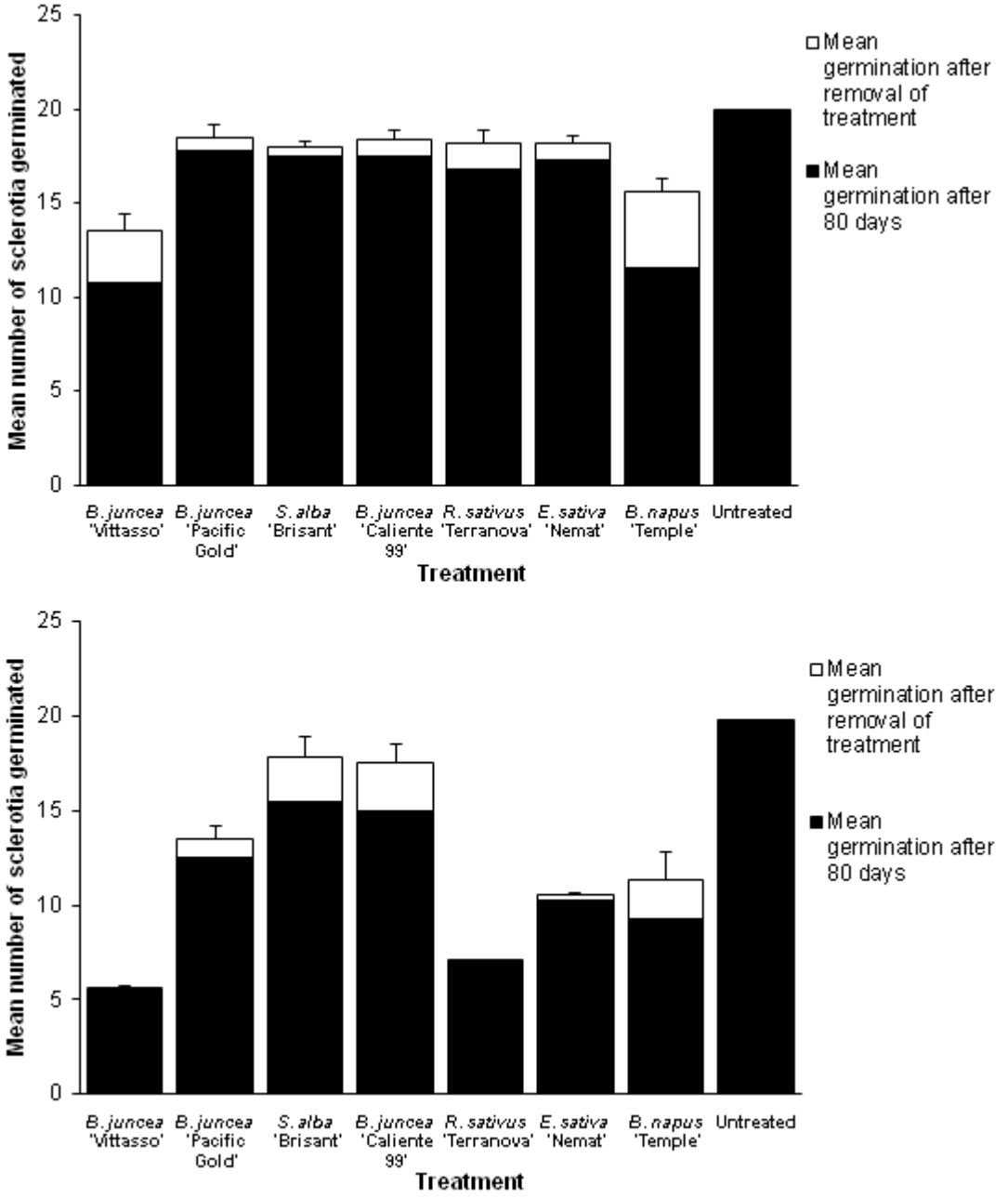

Figure 6. The effect of six biofumigant plants and *Brassica napus* 'Temple' on mean number of *Sclerotinia sclerotiorum* isolate L6 sclerotia germinated (out of 30) in the *in vitro* experiments using either a) 1 g or b) 2 g plant material after 80 days (black bars), and then after four weeks following the removal of each treatment (white bars). Error bars indicate SEM for mean germination after removal of treatment